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Treating insect-bite hypersensitivity in horses with active vaccination against IL-5

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Abstract: Background Insect-bite hypersensitivity is the most common allergic dermatitis in horses. Excoriated skin lesions are typical symptoms of this seasonal and refractory chronic disease. On a cellular level, the skin lesions are characterized by massive eosinophil infiltration caused by an underlying allergic response. Objective To target these cells and treat disease, we developed a therapeutic vaccine against equine IL-5 (eIL-5), the master regulator of eosinophils. Methods The vaccine consisted of eIL-5 covalently linked to a virus-like particle derived from cucumber mosaic virus containing the tetanus toxoid universal T-cell epitope tt830-843 (CMVTT). Thirty-four Icelandic horses were recruited and immunized with 400 g of eIL-5–CMVTT formulated in PBS without adjuvant (19 horses) or PBS alone (15 horses). Results The vaccine was well tolerated and did not reveal any safety concerns but was able to induce anti-eIL-5 autoantibody titers in 17 of 19 horses. This resulted in a statistically significant reduction in clinical lesion scores when compared with previous season levels, as well as levels in placebo-treated horses. Protection required a minimal threshold of anti-eIL-5 antibodies. Clinical improvement by disease scoring showed that 47% and 21% of vaccinated horses reached 50% and 75% improvement, respectively. In the placebo group no horse reached 75% improvement, and only 13% reached 50% improvement. Conclusion Our therapeutic vaccine inducing autoantibodies against self IL-5 brings biologics to horses, is the first successful immunotherapeutic approach targeting a chronic disease in horses, and might facilitate development of a similar vaccine against IL-5 in human subjects.

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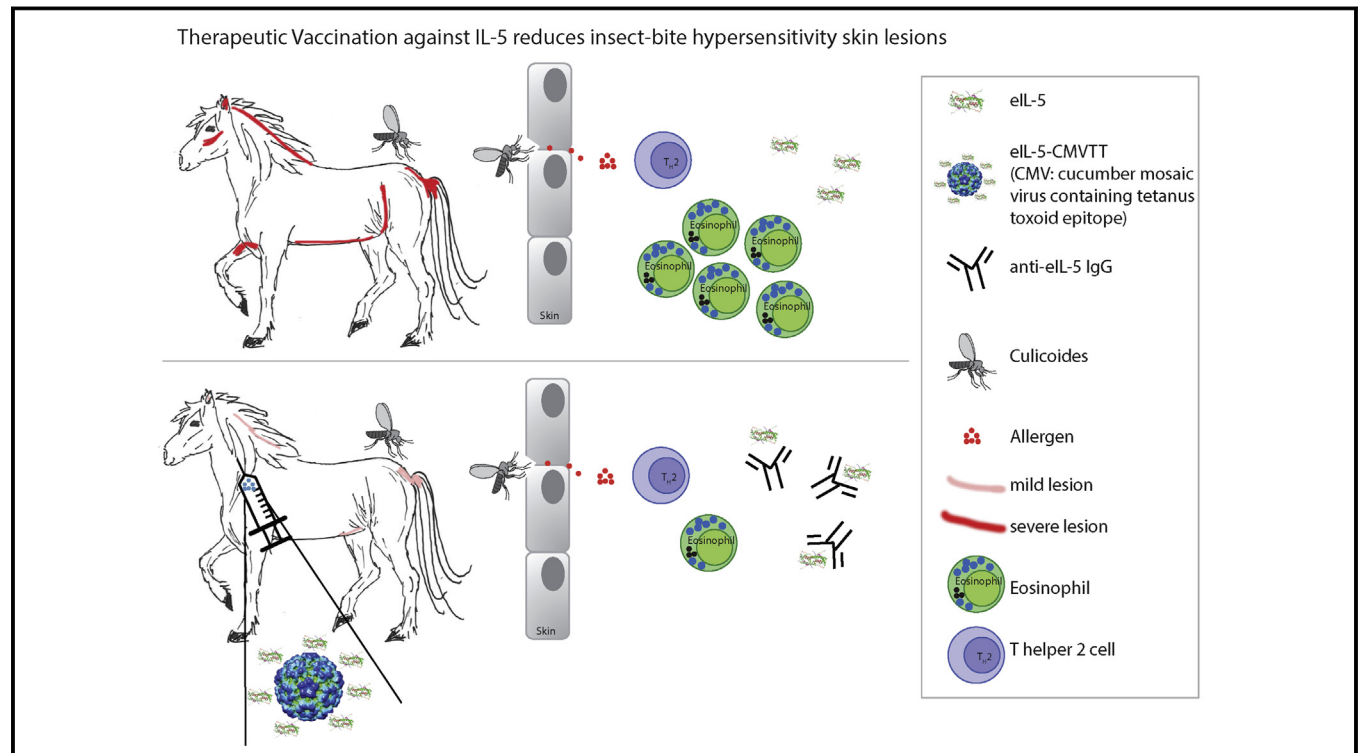
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Treating insect-bite hypersensitivity in horses with active vaccination against IL-5

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GRAPHICAL ABSTRACT



Background: Insect-bite hypersensitivity is the most common allergic dermatitis in horses. Excoriated skin lesions are typical symptoms of this seasonal and refractory chronic disease. On a

cellular level, the skin lesions are characterized by massive eosinophil infiltration caused by an underlying allergic response.

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Animal Health Limited. V. Fettelschoss reports personal fees from Evax AG. F. Thoms is an employee of HypoPet AG (formerly Saiba Biotech GmbH). A. Zeltins reports a grant from EVAX AG and employment from the Latvian Biomedical Research and Study Center, Riga, Latvia. T. M. Kündig reports grants from CTI, Sinergia SNF, and MERIT and personal fees from Evax AG. M. F. Bachmann reports personal fees from Evax AG and personal fees and nonfinancial support from Saiba GmbH and has a patent (PCT/EP2016/071078) pending to Benchmark Animal Health Limited. The rest of the authors declare that they have no relevant conflicts of interest.

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Objective: To target these cells and treat disease, we developed a therapeutic vaccine against equine IL-5 (eIL-5), the master regulator of eosinophils.

Methods: The vaccine consisted of eIL-5 covalently linked to a virus-like particle derived from cucumber mosaic virus containing the tetanus toxoid universal T-cell epitope tt830-843 (CMV_{TT}). Thirty-four Icelandic horses were recruited and immunized with 400 µg of eIL-5-CMV_{TT} formulated in PBS without adjuvant (19 horses) or PBS alone (15 horses).

Results: The vaccine was well tolerated and did not reveal any safety concerns but was able to induce anti-eIL-5 autoantibody titers in 17 of 19 horses. This resulted in a statistically significant reduction in clinical lesion scores when compared with previous season levels, as well as levels in placebo-treated horses.

Protection required a minimal threshold of anti-eIL-5 antibodies. Clinical improvement by disease scoring showed that 47% and 21% of vaccinated horses reached 50% and 75% improvement, respectively. In the placebo group no horse reached 75% improvement, and only 13% reached 50% improvement.

Conclusion: Our therapeutic vaccine inducing autoantibodies against self IL-5 brings biologics to horses, is the first successful immunotherapeutic approach targeting a chronic disease in horses, and might facilitate development of a similar vaccine against IL-5 in human subjects. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: Allergic dermatitis, eosinophils, vaccination

Insect-bite hypersensitivity (IBH) of horses, also known as sweet itch, summer eczema, kasen, or Queensland itch, manifests as chronic relapsing seasonal allergic dermatitis caused by the biting of insects of the genus *Culicoides*.¹⁻⁵

Culicoides species midges are found in various areas of the world.⁶⁻⁸ The incidence of IBH correlates strongly with the geographic distribution of *Culicoides* species, showing the highest incidence in some parts of Australia (60%).⁹⁻¹¹ Overall, approximately 10% of all horses worldwide are affected by IBH,¹²⁻¹⁵ and in principle, all breeds can succumb to the allergic disease. During warmer months of the year, IBH-affected horses experience hairless, weeping, and sometimes even ulcerative lesions caused by inflammation and severe itching. Lesions are characterized by hyperkeratosis, lichenification of the skin, bleeding, swelling, scales, and crust formation. Histologic hallmarks of IBH lesions are thickening of the stratum corneum, epidermis, and dermis, with abundant fibrosis in the latter.⁹ Commonly, secondary infections with bacteria, mites, and fungi can cause further local irritation, enhancing lesion formation. Although IBH was first described in 1840 and is currently the best characterized allergic disease in horses, treatment options are still poor, and currently, no satisfactory treatment of IBH is available.^{9,16}

IBH is classified as IgE-dependent type I allergy,¹⁷ with a strong involvement of type IV allergic hypersensitivity reactions.^{4,18} In addition to IgE-mediated cross-linking of FcεRI on mast cells and basophils with subsequent histamine release, type I allergies also consist of a late-phase reaction with eosinophilia and recruitment of eosinophils into the allergic site. Intradermal injection of *Culicoides* species extract into IBH-affected horses leads to recruitment of T_H2 cells and

Abbreviations used

CMV:	Cucumber mosaic virus
CMV _{TT} :	Cucumber mosaic virus containing the tetanus toxoid universal T-cell epitope tt830-843
eIL-5:	Equine IL-5
HRP:	Horseradish peroxidase
IBH:	Insect-bite hypersensitivity
ISI:	Insect-bite hypersensitivity severity index
MALDI:	Matrix-assisted laser desorption ionization
MS/MS:	Tandem mass spectrometry
PBST:	PBS-Tween 0.1% (vol/vol)
VLP:	Virus-like particle

eosinophils to the injection site.¹⁹ Moreover, in addition to the role of IgE and type I allergy in the setting of IBH, an involvement of cell-mediated type IV allergic reactions, also called delayed-type hypersensitivity, has been discussed in recent years.¹⁶

Type IV allergies can be divided into 4 subgroups: (ie, types IVa, IVb, IVc, and IVd) depending on the cell types involved. Type IVb allergy is strongly associated with IL-5–producing T_H2 cells, eosinophilia, and eosinophilic type of inflammation²⁰ and reflects the clinical manifestations of skin inflammation in IBH, which are characterized by edema and eosinophil accumulation in perivascular clusters in deeper parts of the dermis.²¹ These enhanced eosinophils counts represent the predominant inflammatory cell type accumulating in IBH lesions.^{21,22} Activated eosinophils release granule enzymes and other effector molecules, such as histamine, eosinophil-derived neurotoxin, cysteinyl leukotrienes, and major basic protein. Major basic protein triggers degranulation of mast cells and basophils, thereby further enhancing allergic symptoms.²³ Cysteinyl leukotrienes derived from arachidonic acid are inflammatory mediators contributing on multiple levels to an allergic reaction, thereby making an eosinophil a very effective cell type to cause strong allergic symptoms during late-phase and delayed-type reactions. Therefore eosinophil degranulation can mediate hyperreactivity, inflammation, and local tissue damage, thus potentially driving the pathology of IBH.²⁴

To dampen eosinophil activity, we decided to target the lineage-specific eosinophil master regulator IL-5, which is a T_H2-type cytokine produced mainly by T_H2 cells and also by mast cells. Eosinophil development in the bone marrow is critically dependent on IL-5.^{25,26} Moreover, eosinophil release into blood circulation is mediated by IL-5, and the cytokine plays a key role in eosinophil activation.²⁷ Also, IL-5 increases survival of tissue-resident inflammatory eosinophils.²⁸ Mice either vaccinated against murine IL-5 or IL-5–deficient knockout mice show strongly reduced levels of blood eosinophils and eosinophil-mediated inflammation.^{26,29} Furthermore, mepolizumab, a humanized anti-IL-5 mAb, significantly reduced circulating eosinophil counts in human subjects and has shown clinical efficacy in patients with eosinophil-mediated diseases, such as eosinophil-mediated asthma and hypereosinophilic syndrome.³⁰⁻³⁴ Thus blocking IL-5 can lead to effective control of IBH.

Instead of targeting IL-5 with mAbs,³⁵ which is not a realistic approach because of the weight of a horse, we developed a

virus-like particle (VLP)-based therapeutic vaccine that induces IL-5 autoantibodies in the host. VLPs are supramolecular structures composed of multiple copies of 1 (or more) recombinantly expressed structural viral proteins, which spontaneously assemble into capsid particles but lack genetic information and therefore do not have replicative capacity, which is an important safety aspect. The highly repetitive surface, particulate structure, and induction of innate immunity through pathogen-associated molecular patterns is responsible for induction of fulminant B- and T-cell responses.^{36,37} VLPs can be derived from a large number of viruses, including bacteriophages and plant viruses,^{38,39} and are used for the development of a wide range of different prophylactic and therapeutic vaccines.

Here we used a recently developed and novel VLP platform based on the cucumber mosaic virus containing the tetanus toxoid universal T-cell epitope tt830-843 (CMV_{TT}) to enhance T_H cell-dependent IgG responses for antigens displayed on the VLP surface.⁴⁰ We show that a vaccine consisting of equine IL-5 (eIL-5) chemically linked to CMV_{TT}-VLPs induces potent anti-eIL-5 antibody responses, which improve symptoms of IBH in horses.

METHODS

Horses and clinical study

All study horses were Icelandic horses and privately held by their owners. All clinical studies were approved by the respective cantonal veterinary authorities. All horse owners signed informed consent. The clinical study was performed as a double-blind, placebo-controlled randomized trial; neither the person that performed the clinical trial nor the horse owners knew the group identity of the horses. The independent vaccine filler has randomized the study and was not involved in any clinical part of the study.

IBH lesion scoring

For IBH symptom scoring, all locations (tail, mane, belly, flank, face, ear, leg, and the like) at which IBH lesions occurred were recorded. Each location was divided into 3 parts: up, middle, and down. Furthermore, according to the number of lesions, each location was classified as light or strong, depending on the frequency of lesions within the particular area. Depending on how many parts are affected (up/middle/down) and how many lesions per location were found (light/strong), 1 to 4 points were scored (1 point = one part affected, lesion light; 4 points = all 3 parts affected, lesion strong). Moreover, these locations were classified for 6 further properties: size (diameter), blood (occurrence of bleeding), hair loss, scales, crust, and lichenification/swelling. For all these properties, 1 to 4 points were also scored. Lesion size was divided into 1 point (<0.5 cm), 2 points (0.5 ≤ < 1 cm), 3 points (1 ≤ < 2 cm), and 4 points (≥ 2 cm). The "blood" criteria served to distinguish between intact epidermis (1 point) or whether mild (2 points), moderate (3 points), or severe (4 points) bleeding occurred. Hair loss was divided into mild (1 point), moderate (2 points), severe (3 points), and absence of hair (4 points). Scales were divided into none (1 point), tiny and few (2 points), moderate and midsized (3 points), and many and big (4 points) scales. Crust was divided into none (1 point), tiny (2 points), half (3 points), and total (4 points). Lichenification and/or swelling were divided into none (1 point), mild (2 points), moderate (3 points), and severe (4 points). Additionally, if the sheath or udder was swollen, a minimum of 5 or a maximum of 20 points were scored: grade 1 (5 points), grade 2 (10 points), grade 3 (15 points), and grade 4 (20 points). Finally, all points were summed to produce the IBH symptom (or lesion) score.

The Δ average lesion score was calculated for placebo-treated and vaccinated horses as follows. Lesion scores for all horses were recorded monthly during the observation period within each IBH season. For each horse, the average lesion score was calculated per season. The Δ average lesion score was defined by subtracting the pretreatment season average lesion

score by the treatment season average lesion score. The average of the resulting differences was calculated and is reported here as Δ mean average lesion score.

Blood withdrawal

Blood was collected from the jugular vein at the intersection of the proximal to median third of the neck.

Blood analysis with IDEXX Diavet and production of horse sera and plasma

For differential blood analysis and determination of IgE levels against *Culicoides* species, blood was collected in tubes provided by IDEXX Diavet (Freienbach, Switzerland). Differential blood analysis was done with fresh EDTA blood. IgE levels against insects were determined in serum, EDTA plasma, and Lithium Heparin plasma; tubes were centrifuged at 3000 rpm for 10 minutes, and serum or plasma was transferred into fresh tubes. IgE-specific *Culicoides* species ELISA was performed by IDEXX Diavet with *Culicoides* species whole-body extract.

Detection of recombinant eIL-5 by anti-IL-5 ELISA

Maxisorp 96-well ELISA plates (Nunc; Thermo Fisher) were coated overnight with 50 μ L of anti-His antibody at 0.5 mg/L. Plates were washed 3 times with PBS-Tween 0.1% (vol/vol; PBST) and then blocked with Superblock (Thermo Scientific, Waltham, Mass) for 1 hour at 37°C. Plates were washed twice with PBST, and purified recombinant eIL-5-C-His was titrated starting with addition of 10 mg/L diluted 1:3 and incubated for 1 hour. Blank wells contain Superblock without recombinant protein. Then plates were washed 3 times with PBST and 1 ng/mL anti-eIL-5 antibody (eIL-5 affinity purified polyclonal antibody, catalog no. AF2470; R&D Systems, Abingdon, United Kingdom) was added and incubated at room temperature for 2 hours. Plates were washed subsequently 3 times with PBST and incubated with a secondary anti-goat IgG conjugated with horseradish peroxidase (HRP; dilution 1:2000) at room temperature for 30 minutes. Plates were again washed 3 times with PBS, and 50 μ L/well developing solution (TMB) was added. After 2 minutes of reaction at room temperature, the ELISA was stopped with 25 μ L/well at 5% H₂SO₄. Absorbance was measured at 450 nm on a Tecan M200 spectrophotometer (Tecan, Grödig, Austria).

eIL-5 levels in horse serum

ELISA was conducted according to the manufacturer's manual (R&D Systems).

Anti-CMV_{TT} and anti-IL-5 antibody titer determination

Maxisorp 96-well ELISA plates (Nunc) were coated overnight with purified eIL-5-C-His or purified CMV (5 g/L). Plates were washed 3 times with PBST and blocked with Superblock (Thermo Scientific) for 2 hours at room temperature. Then plates were washed 3 times with PBST, and 3-fold dilutions of 1:10 diluted horse sera (in Superblock) were added and incubated at room temperature for 2 hours. Plates were washed subsequently 3 times with PBST and incubated with anti-equine IgG (1:2000) conjugated with HRP (Jackson ImmunoResearch, West Grove, Pa) at room temperature for 30 minutes. Plates were again washed 4 times with PBS, and developing solution (TMB) was added. After approximately 2 minutes of reaction at room temperature, the ELISA was stopped with 5% H₂SO₄. Absorbance was measured at 450 nm (OD₄₅₀) on a Tecan M200 spectrophotometer (Tecan). The antibody titer as OD₅₀ was calculated (serum dilution on a logarithmic scale where OD₄₅₀ was half maximal). All antibody titers are calculated with naive serum subtracted on logarithmic scales and presented as Δ OD₅₀ values. Titers of less than 10 (and including 10) were considered as background.

IBH lesion biopsy and hematoxylin and eosin staining

Punch biopsy specimens (6 mm) from lesions of IBH-affected horses were stained with hematoxylin and eosin and CD3 (Vetsuisse Faculty, Zurich, Switzerland).

Cloning, expression, and purification of recombinant eIL-5

The DNA sequence encoding mature eIL-5 (*Equus caballus*; UniProt O02699) was generated by means of gene synthesis. In addition, a 3-amino-acid linker (GGC) was added C-terminally. This insert was flanked by 5' *NdeI* and 3' *XhoI* and integrated into pET 42b (+), containing a hexa His-tag (to facilitate purification) and an in-frame stop codon. The resulting eIL-5 fusion protein was expressed in *Escherichia coli* BL21 (DE3) cells. Overnight cultures were grown and diluted in LB medium containing 50 mg/L kanamycin. Isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 1 mmol/L when an OD₆₀₀ of culture reached 0.7. The culture was harvested by means of centrifugation 4 hours after induction, and the pellet was resuspended in 100 mmol/L Tris/HCl (pH 8.0) at 4°C and 1 mmol/L EDTA. Inclusion bodies were prepared, and insoluble eIL-5 was solubilized in denaturing buffer (6 mol/L GdmCl, 20 mmol/L imidazole, and 100 mmol/L Tris-HCl [pH 8]). After centrifugation for 20 minutes at 20,000g, the supernatant containing denatured, soluble, recombinant eIL-5 was purified through the His-tag by using Ni-NTA (Ni-NTA Sepharose 6 Fast Flow, Amersham, Piscataway, NJ, or NiNTA Superflow, Qiagen, Hilden, Germany) column and eluted by using 6 mol/L GdmCl, 100 mmol/L NaH₂PO₄, and 10 mmol/L Tris-HCl (pH 4.5) with subsequent dialysis to pH 8.0. Accordingly, eIL-5 was refolded by means of sequential dialysis against the following buffers at pH 8.5 at 4°C: buffer 1 (2 mol/L urea, 50 mmol/L NaH₂PO₄, 5 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione, 0.5 mol/L arginine, and 10% glycerol), buffer 2 (50 mmol/L NaH₂PO₄, 5 mmol/L reduced glutathione, 0.5 mmol/L oxidized glutathione, 0.5 mol/L arginine, and 10% glycerol), buffer 3 (50 mmol/L NaH₂PO₄ and 10% glycerol), and buffer 4 (PBS). Finally, refolded protein was concentrated by using Amicon Centrifugal Filters (Ultrafree-15 [Millipore, Temecula, Calif], 10 kDa MWCO) and purified on a HiLoad 26/60 Superdex 75 prep grade (GE Healthcare, Fairfield, Conn) with PBS buffer. Protein concentrations were determined by using the Bradford assay to BSA standard.

Circular dichroism spectroscopy of purified eIL-5-C-His

The far-UV circular dichroism spectrum of purified eIL-5-C-His (in PBS) was measured on a J-710 spectropolarimeter (Jasco, Easton, Md) at 25°C by using a 1-mm cuvette. After correction for the buffer spectrum, ellipticity was converted to mean residue ellipticity, as previously described.⁴¹

Coupling of eIL-5 to CMV_{TT}

VLP-CMV_{TT} derived from CMV and containing the tetanus toxoid universal T-cell epitope tt830-843 was expressed in *Escherichia coli* by using the expression plasmid pET28e, as previously described.⁴⁰ The CMV concentration was determined by using the Bradford assay to a BSA standard. CMV was reacted with a 10-fold molar excess of the heterobifunctional cross-linker succinimidyl-6-(β -maleimidopropionamido)hexanoate in 20 mmol/L NaP/2 mmol/L EDTA, pH 7.5, at 25°C (Pierce, Rockford, Ill). Unreacted cross-linker was removed by means of passage over a PD-10 desalting column (GE Healthcare). Recombinant, purified, and refolded eIL-5-C-His was reduced for 1 hour with an equimolar amount of tri(2-carboxyethyl)phosphine hydrochloride in PBS (pH 8.0) to reduce the cysteine residue contained in the linker. The reduced eIL-5-C-His was then mixed with the derivatized CMV_{TT}-VLPs at a molar ratio of CMV_{TT} monomer to eIL-5-C-His protein monomer of 1:2 and incubated for 4 hours at 22°C in PBS (pH 7.4) to allow cross-linking.

SDS-PAGE and Coomassie staining

Separation of proteins with 4-12% Bis-Tris SDS-PAGE gels was done with a prestained SeeBlue marker (Thermo Fisher) and MES buffer (NuPAGE; Novex, Invitrogen Life Technologies, Grand Island, NY) at 200 V for 55 minutes. For coupling analysis, equimolar amounts of CMV_{TT}, eIL-5-C-His, and eIL-5-C-His-CMV_{TT} VLPs were separated by using SDS-PAGE, 4-12% B/T gel (NuPAGE, Invitrogen), and MES buffer at 200 V for 55 minutes. Subsequently, the gel was stained with Coomassie Blue (0.025% Coomassie Brilliant Blue R-250, 40% methanol, and 10% acetic acid) and destained with destainer (40% methanol and 10% acetic acid). The coupling efficiency (ie, moles of CMV-IL-5/ moles of CMV monomer [total]) was estimated by using densitometric analysis of the Coomassie Blue-stained SDS-PAGE with ImageJ software (National Institutes of Health, Bethesda, Md).

Western blotting

CMV_{TT}, eIL-5-C-His, and IL-5-C-His-CMV_{TT} vaccine were separated by using SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was blocked for 1 hour with 5% (wt/vol) BSA powder in PBST and then incubated with 10 mL of 1:1000 diluted anti-His antibody (Penta-His Antibody, BSA-free, mouse monoclonal IgG₁; Qiagen) in 1% BSA (wt/vol) powder in PBST. The membrane was washed with PBST for 15 minutes and then incubated for 1 hour with 10 mL of 1% (wt/vol) BSA in PBST containing an anti-mouse IgG antibody conjugated with HRP at a dilution of 1:10,000. The membrane was washed for 15 minutes in PBS and developed with ECL (Amersham Pharmacia, Sweden) and exposed to photographic film.

Vaccine administration and immunization regimen

Horses (n = 19) were injected subcutaneously on days 0, 28, 56, and 84 with 400 μ g of eIL-5-C-His-CMV_{TT} VLPs in 1000 μ L of PBS without additional adjuvants to generate self-reactive antibodies to eIL-5. All horses received a booster immunization on day 140. Placebo-treated horses (n = 15) received PBS instead at all respective time points.

Parasite load

Fresh feces was collected from horses before and after vaccination and was analyzed at IDEXX Diavet by using the combined sediment/float method, distinguishing gastrointestinal nematodes, coccidian oocysts, and tapeworm eggs/body parts. For each time point (before and after vaccination), a value of 1 was allocated to horses with helminths, and a value of 0 was allocated to horses without helminths. Mean helminth values were calculated for eIL-5-CMV_{TT}-vaccinated and placebo-treated horses. The Δ helminth load for placebo-treated or vaccinated horses was calculated by subtracting the mean value after treatment from the mean value before treatment.

Statistics

All graphs comparing vaccinated horses versus placebo-treated horses show means and SEMs. For statistical analysis, vaccine horses versus placebo horses were compared by using an unpaired 2-tailed Student *t* test. *P* values of less than .05 were considered to be statistically significant.

RESULTS

Eosinophil counts but not IgE levels correlate with disease severity

To compare the influence of levels of *Culicoides* species-specific IgE versus eosinophil counts, we clinically scored signs of IBH (by score sheet, see Fig E1 in this article's Online Repository at www.jacionline.org) in affected horses for a whole season and analyzed blood for blood eosinophil counts and *Culicoides* species-specific IgE levels using a commercial serologic test. Blood was taken in the season before vaccination. Eleven of the

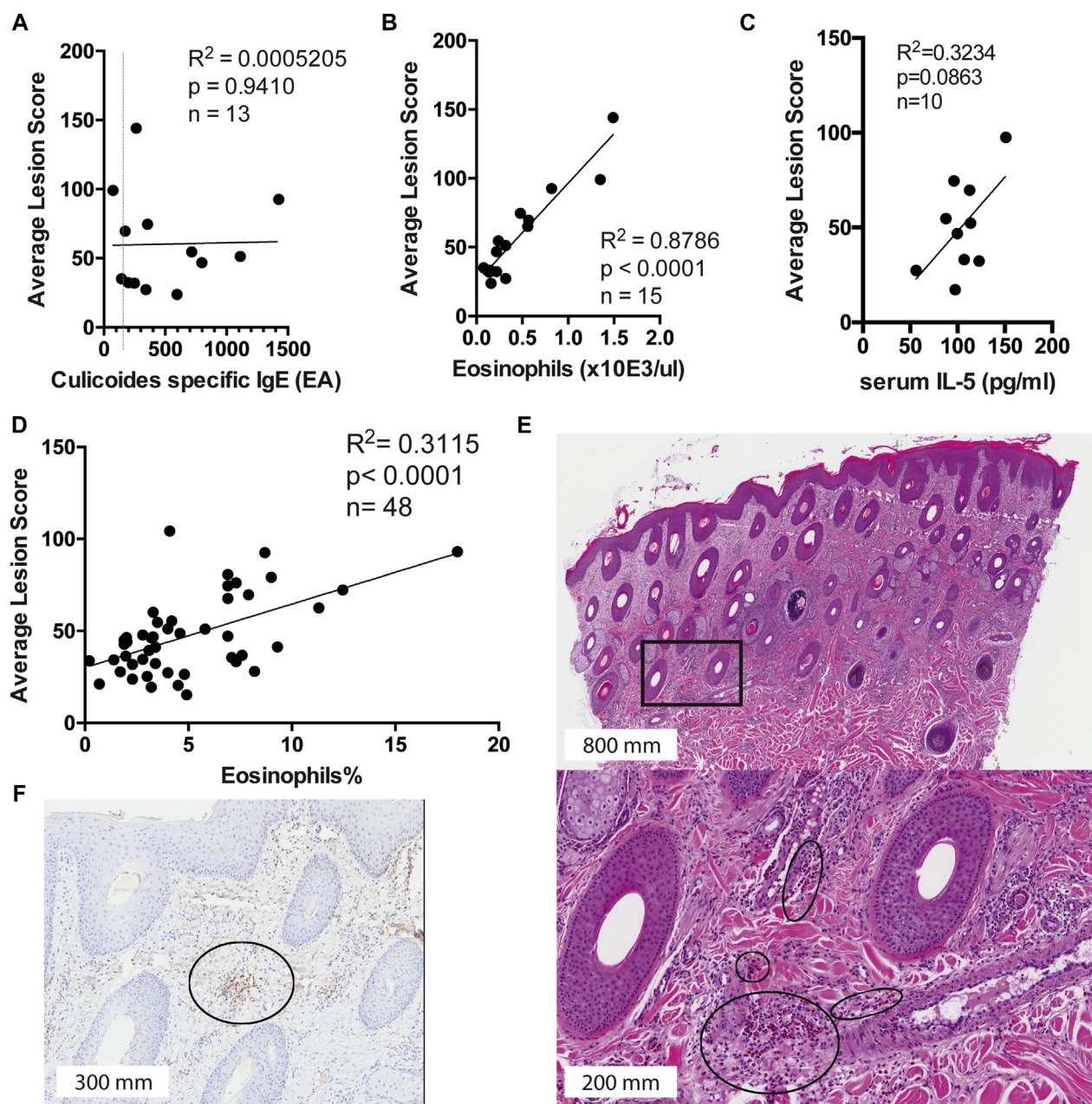


FIG 1. Diagnostics and characterization of IBH. **A-D**, Correlation of IBH lesion scores to blood parameters. IBH lesion scores were determined in biweekly to 4-week intervals during a whole season (April until October). Average lesion scores per horse were generated. EDTA blood and serum were taken once during the season with high symptoms (August). Eosinophil levels (EDTA blood) and *Culicoides* species-specific IgE levels (serum) were quantified by IDEXX Diavet. Correlation between average lesion scores and *Culicoides* species-specific IgE levels (IgE levels >150 ELISA absorbance units [EA] are considered allergic; dotted gray line; Fig 1, A), blood eosinophil count (Fig 1, B), serum IL-5 levels (quantified by means of ELISA; Fig 1, C), and blood eosinophil counts in a larger cohort (Fig 1, D) are shown. **E** and **F**, IBH skin biopsy. A representative skin biopsy specimen of a fresh lesion from an IBH-affected horse. Fig 1, E, Eosinophils in an IBH skin biopsy specimen. Hematoxylin and eosin staining was used. An overview (scale bar = 800 μm , upper panel) and enlarged section (scale bar = 200 μm , lower panel) are shown. Eosinophil-rich regions in the enlarged section are marked by circles. Fig 1, F, T cells in an IBH biopsy specimen. CD3 staining was used. An overview (scale bar = 300 μm) is shown. A CD3⁺-rich region is marked by a circle.

13 tested IBH-affected horses had *Culicoides* species-specific IgE levels of greater than the cutoff of 150 ELISA absorbance units. However, 1 horse with very severe clinical signs of IBH (average IBH score, 99) had levels less than the cutoff, and

overall, IgE levels did not correlate with average scores of seasonal IBH severity (Fig 1, A). In contrast, there was a strong positive correlation between average seasonal IBH scores and eosinophil count in blood at time points of active disease

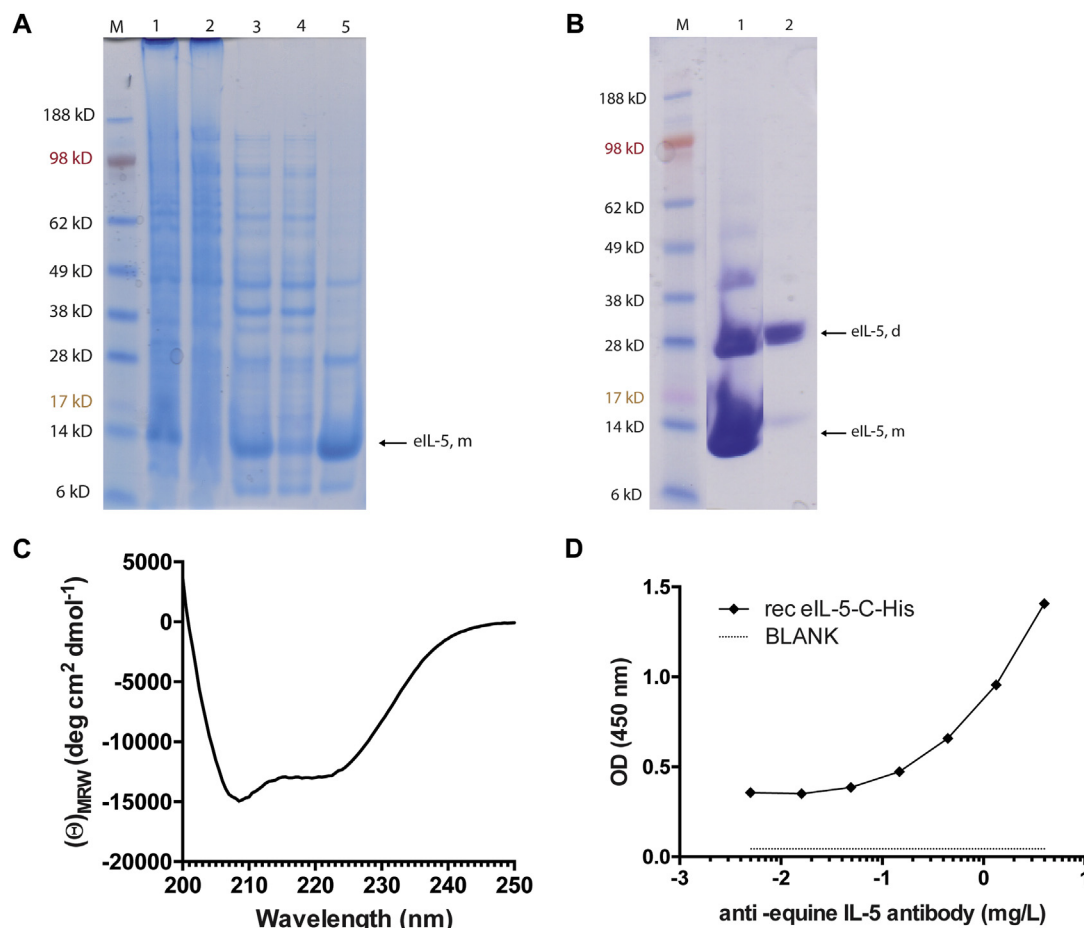


FIG 2. Production and characterization of eIL-5 protein. **A**, SDS-PAGE analysis of purified eIL-5-C-His. Samples from various stages of the inclusion body preparation and purification were applied to a 4-12% B/T gel and run under reducing conditions. Proteins were stained with Coomassie Blue. *Lane M*, Size marker; *lane 1*, lysate (sample A); *lane 2*, soluble fraction (sample B); *lane 3*, solubilized inclusion bodies (sample C); *lane 4*, flow through (unbound material, sample D); and *lane 5*, pooled eIL-5 monomer (*eIL-5, m*) eluate from the Ni-NTA column (sample E). **B**, SDS-PAGE of refolded recombinant eIL-5-C-His. An aliquot of purified eIL-5-C-His was separated on a 4-12% B/T gel and run under nonreducing conditions (+SDS, no dithiothreitol, no heating). Proteins were stained with Coomassie Blue. *eIL-5, d*, eIL-5 dimer; *eIL-5, m*, eIL-5 monomer. *Lane M*, Size marker; *lane 1*, pooled denatured eluate from the Ni-NTA column; *lane 2*, refolded and homodimer enriched eIL-5-C-His. **C**, Far-UV circular dichroism spectrum of purified eIL-5-C-His. The spectrum demonstrates that eIL-5-C-His was folded. **D**, Detection of purified eIL-5-C-His with commercial anti-eIL-5 antibody. Anti-His antibody-coated ELISA plates were incubated with purified eIL-5-C-His and detected with a commercially available anti-eIL-5 antibody.

(Fig 1, B). Moreover, serum IL-5 levels at the same time point also correlated to average seasonal IBH scores, thereby indicating a link between levels of eIL-5 and IBH-associated eosinophilia (Fig 1, C). The correlation of eosinophil blood counts with IBH scores was also seen when a second larger cohort of horses was assessed (Fig 1, D). A representative biopsy of a skin lesion stained with hematoxylin and eosin is shown in Fig 1, E, confirming recruitment of blood eosinophils to the skin. In addition to the remarkable eosinophil infiltrates, the most abundant other cell type also associated with skin pathology was the CD3⁺ T cell (Fig 1, F). Furthermore, the typical hyperkeratosis accompanied by acanthosis can be found, fulfilling the histologic hallmarks of an IBH lesion (Fig 1, E).

Taken together, blood eosinophil counts correlate strongly with IBH severity scores and therefore might not only be useful to monitor IBH severity but also are an attractive target in IBH

therapy. Consequently, a therapy treating eosinophilia is likely to influence the magnitude of disease severity.

Production of therapeutic vaccine

To induce autoantibodies against eIL-5, we coupled eIL-5 to VLPs, creating a hapten-carrier complex and thus bypassing B-cell unresponsiveness against self IL-5 by introducing VLP-specific intermolecular T-cell help.⁴²

Recombinant eIL-5 with a C-terminal linker containing a free cysteine residue and a His-tag (eIL-5-C-His) was produced in *E coli* and purified by means of affinity chromatography (Fig 2, A), refolded, and polished by using size-exclusion chromatography. Because biologically active native IL-5 is a disulfide-linked homodimer, the ability of purified recombinant eIL-5-C-His to form dimers after refolding was assessed by using

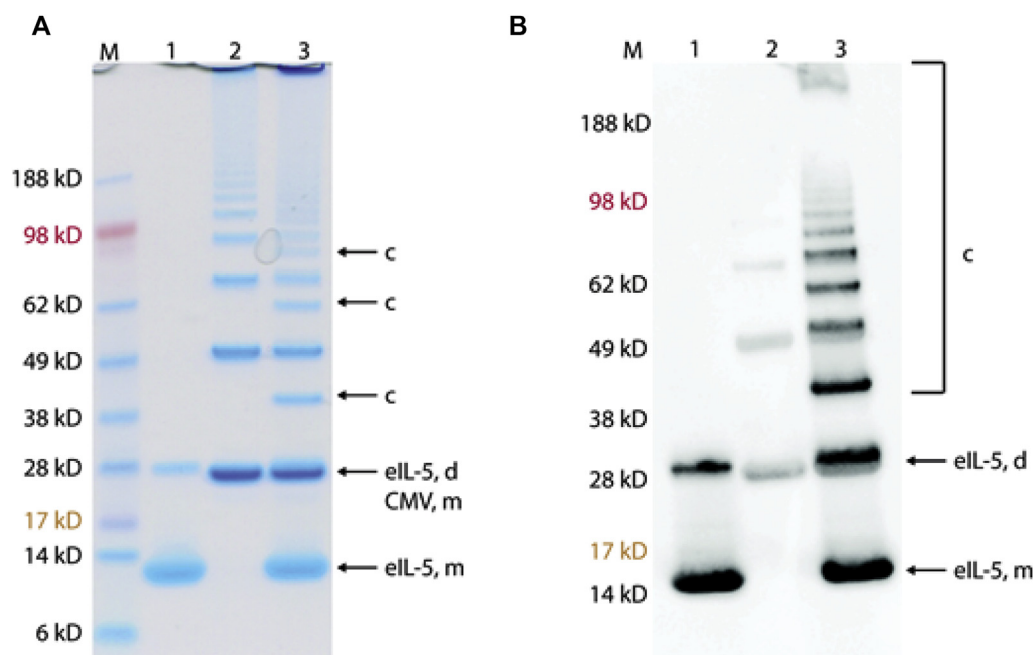


FIG 3. Vaccine production and analysis of the coupling reaction of eIL-5-C-His to CMV_{TT}. **A and B:** Lane M, Size marker; lane 1, tri(2-carboxyethyl)phosphine hydrochloride (TCEP)-activated eIL-5-C-His; lane 2, CMV-VLP after derivatization with the chemical cross-linker succinimidyl-6(β-maleimidopropionamido)hexanoate (SMPH); lane 3, eIL-5-C-His-CMV_{TT} coupling reaction. eIL-5-C-His, CMV_{TT}, and eIL-5-CMV_{TT} were loaded in an equimolar manner to compare coupling efficacy. **A,** SDS-PAGE. Proteins were stained with Coomassie Blue. c, Coupling; CMV, m, CMV_{TT} monomer; eIL-5, d, eIL-5 dimer; eIL-5, m, eIL-5 monomer. **B,** Western blotting with α-His antibody staining. c, Coupling; eIL-5, d, eIL-5 dimer; eIL-5, m, eIL-5 monomer.

SDS-PAGE performed under nonreducing conditions (Fig 2, B, lane 2). As judged by the molecular mass of approximately 28 kDa, eIL-5-C-His was indeed dimeric in nature, indicating conservation of the native tertiary structure. Far-UV circular dichroism spectroscopy confirmed that purified eIL-5-C-His was folded and, as expected and indicated by the minima at 208 and 222 nm, adopted a mostly α-helical secondary structure (Fig 2, C). Furthermore, a commercially available anti-eIL-5 mAb also recognized the refolded dimeric eIL-5 (Fig 2, D). In the native structure of the IL-5 dimer, the 2 monomers are linked by 2 intermolecular disulfide bridges (between Cys44 of one monomer and Cys86 of the other and *vice versa*), leading to a head-to-tail positioning of the 2 monomers with respect to each other.⁴³ In particular, linkage of 2 monomers by 2 intermolecular disulfide bridges was analyzed by using liquid chromatography–matrix-assisted laser desorption ionization (MALDI)–tandem mass spectrometry (MS/MS) after trypsin digestion of eIL-5-C-His. MS/MS fractions of mass 2505, 2633, and 2761 m/z showed the typical disulfide fragment pattern 32/2/32, confirming intermolecular linkage of 2 IL-5 monomers through Cys44 to Cys86 (see Fig E2 in this article's Online Repository at www.jacionline.org).

The eIL-5-C-His homodimers were chemically coupled to VLPs derived from the cucumber mosaic virus (CMV_{TT}) through the heterobifunctional cross-linker succinimidyl-6(β-maleimidopropionamido)hexanoate to produce a VLP-based vaccine against IL-5. Derivatization of VLPs shows the typical VLP ladder with CMV monomeric and multimeric subunits (Fig 3, A, lane 2).²⁹ Coupling of CMV subunits with dimeric eIL-5-C-His molecules (Fig 3, A, lane 1) is shown on a reducing SDS-PAGE gel by

additional coupling bands that correspond to the molecular mass of monomeric or dimeric eIL-5 plus monomeric or multimeric CMV subunits (Fig 3, A, lane 3). Successful covalent attachment of eIL-5-C-His to CMV_{TT} was confirmed by using Coomassie staining (Fig 3, A) and Western blotting with an anti-His antibody (Fig 3, B). Coupling efficiency for all batches was between 20% and 50%.

Antibody titer responses on vaccination

Horses were immunized with 400 μg of eIL-5-CMV_{TT} formulated in PBS on days 0, 28, 56, 84, and 140. Placebo-treated horses received PBS. The reasons to use PBS and not CMV_{TT} without IL-5 coupled to a placebo were 2-fold: (1) placebo in registration studies will be PBS and not CMV_{TT} without IL-5 coupled to it, and (2) we planned to immunize the placebo-treated horses in the following season, which would have been impossible if they had been immunized with CMV_{TT} alone because of carrier-induced epitope suppression.⁴⁴ Therefore we decided to use PBS as a control. Antibody titers in horse serum were evaluated monthly after vaccination and were consistently detectable after the second injection (Fig 4). The third and fourth injections maintained antibody responses against eIL-5 (Fig 4, A) and CMV_{TT} (Fig 4, B). Variation between single horses was less pronounced for anti-CMV_{TT} VLP antibody titers (Fig 4, D) than for antibody titers against the self-molecule eIL-5 (Fig 4, C). However, and in contrast to anti-CMV antibodies, anti-IL-5 antibodies can bind to their target antigen, IL-5, in serum. Hence such antigen-antibody complexes might not be detectable by using classical ELISA.

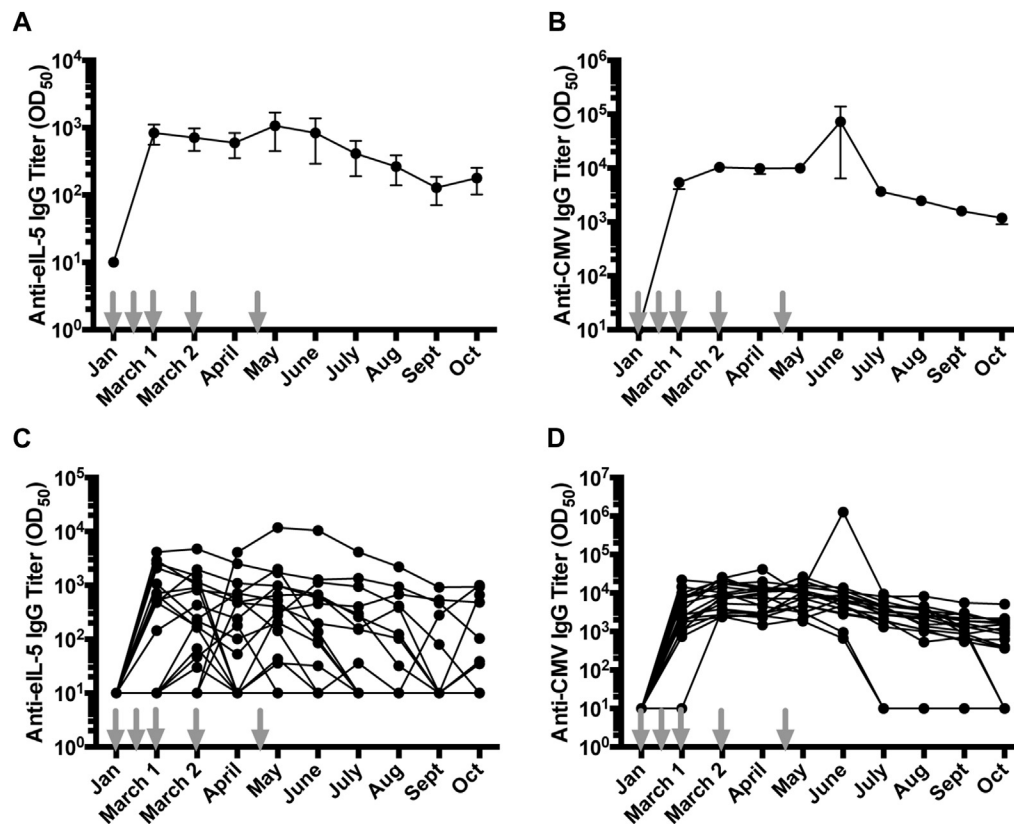


FIG 4. Antibody titer of vaccinated horses against eIL-5 and CMV_{TT}-VLP. Time points of vaccinations are indicated by gray arrows. **A**, Mean antibody titer of anti-eIL-5 IgG. **B**, Mean antibody titer of anti-CMV IgG. **C**, Antibody titer of anti-eIL-5 IgG of individual horses. **D**, Antibody titer of anti-CMV IgG of individual horses.

Mean antibody titers were decreasing toward the end of the season for both eIL-5 and CMV (Fig 4).

Reduced IBH lesion scores in eIL-5–CMV_{TT}-vaccinated horses

Lesion scores of study horses were recorded monthly in the pretreatment season and the subsequent treatment year from March until October. Data were analyzed according to an intention-to-treat analysis, thus including all horses without exclusions. Seasonal progression of average lesion scores of placebo-treated horses were comparable for both seasons (Fig 5, A), whereas average lesion scores of eIL-5–CMV_{TT}-vaccinated horses showed strongly improved clinical signs in the treatment year when compared with those in the pretreatment season (Fig 5, B). When comparing average lesion scores between both seasons for all horses individually, the majority of vaccinated horses showed pronounced lesion reduction in the treatment year. In contrast, most placebo-treated horses did not improve their symptoms (Fig 5, C). Analogous to Psoriasis Area and Severity Index 50 and Psoriasis Area and Severity Index 75 scores, we defined the insect-bite hypersensitivity severity index (ISI) 50 and ISI 75 (IBH severity index). ISI 50 represents a 50% and greater reduction of symptoms, and ISI 75 represents a 75% and greater reduction of symptoms. In total, 47% of vaccinated horses reached an ISI 50, whereas in the placebo group only 13% reached this level of reduction. An ISI 75 was achieved in

21% of the vaccinated horses, whereas none of the placebo-treated horses showed such a pronounced improvement (Fig 5, D). The Δ average lesion scores of eIL-5–CMV_{TT}-vaccinated horses showed a mean reduction in lesion scores of 28.5 ± 4.5 ($n = 19$), whereas the Δ average lesion score in placebo-treated horses showed a mean reduction in lesion score of 11.1 ± 3.7 ($n = 15$). The difference between vaccinated horses and placebo-treated horses was statistically significant, showing a higher improvement in lesion symptoms in eIL-5–CMV_{TT}-vaccinated horses ($P = .0073$; Fig 5, E). Furthermore, anti-eIL-5 serum antibody levels during the IBH season were most likely required to be greater than a certain threshold to be protective rather than showing a linear dependency (Fig 5, F). For further IBH vaccine development, the observed antibody threshold level can be used as a surrogate for clinical efficacy.

Blood eosinophilia

Eosinophil counts for all horses were measured monthly after vaccination or placebo treatment. During the IBH season, blood eosinophil counts of placebo-treated horses peaked in June, whereas lesion scores peaked with a delay of approximately 1 month in July. Mean blood eosinophil counts in placebo-treated horses had been found above maximal standard values from May until October, with a slight decrease in peak levels in July and October (Fig 6, A). In general, blood eosinophil counts of placebo-treated horses correlated well with lesion scores of the skin (Fig 6, B).

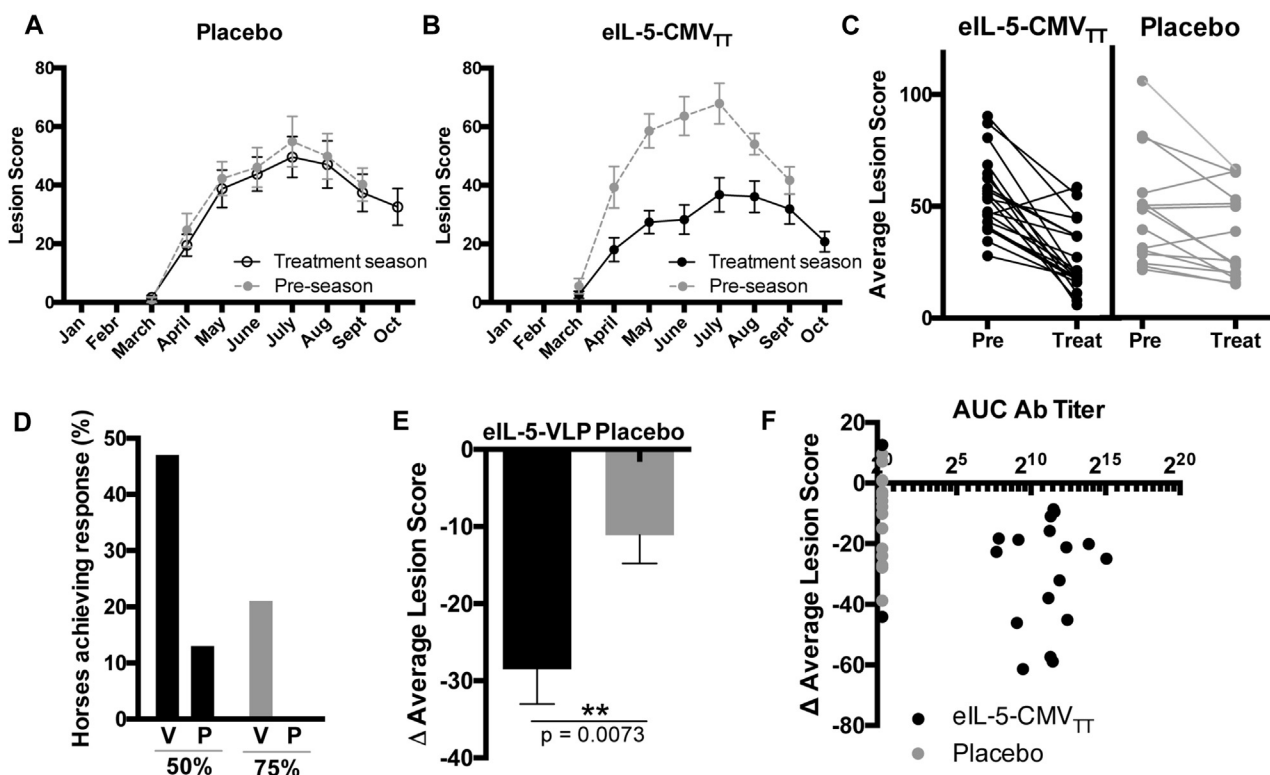


FIG 5. Lesion scores of eIL-5-CMV_{TT}-vaccinated horses improved significantly compared with placebo-treated horses in clinical study. **A** and **B**, Mean lesion score of placebo-treated horses ($n = 15$; Fig 5, **A**) and eIL-5-CMV_{TT}-vaccinated horses ($n = 19$; Fig 5, **B**) followed from March until October in the pre-evaluation year (gray symbols) and treatment year (black symbols). Months with more than 1 measurement show average values. **C**, Average lesion score per horse of the previous season (Pre) versus the treatment season (Treat; black: active, eIL-5-CMV_{TT} vaccinated, $n = 19$; gray: placebo, $n = 15$). **D**, Percentage of horses with 50% (black columns) and 75% (gray columns) lesion score improvement in eIL-5-CMV_{TT}-vaccinated (V) and placebo (P) group. **E**, Δ Mean average lesion score from eIL-5-CMV_{TT}-vaccinated horses (black column, $n = 19$) versus placebo-treated horses (gray column, $n = 15$). **F**, Area under the curve (AUC) anti-eIL-5 antibody (Ab) titer versus Δ average lesion score of placebo-treated horses (gray symbols, $n = 15$) and eIL-5-CMV_{TT}-vaccinated horses (black symbols, $n = 19$). AUC includes all values during the whole treatment season. All graphs include all horses ($n = 34$) analyzed according to intention to treat.

Immunization against eIL-5 did not greatly alter blood eosinophil counts, and there was a much less obvious correlation between blood eosinophil count and lesion scores, indicating that anti-IL-5 antibodies alter eosinophil inflammation in lesions but had little influence on blood eosinophil counts (Fig 6, C).

No increase in parasitic load after eIL-5-CMV_{TT} vaccination

The parasitic load of horses was quantified in excrement at the start and end of the treatment year. No difference between placebo-treated and vaccinated animals was observed when comparing mean helminth load before and after treatment (Fig 7, A) and change in mean helminth load (Fig 7, B). In general, helminth burden in autumn was greater in both the placebo-treated and vaccinated groups, probably because of higher exposure to parasites on grazing land in the summer months.

DISCUSSION

IBH is a frequent and sometimes devastating disease in horses. Here we demonstrate that targeting eosinophils, the major

inflammatory cells in IBH lesions, with active vaccination against the T_H2 cytokine IL-5 strongly improves disease symptoms in affected horses. This is a novel and allergen-independent approach to manage IBH in horses that differs from the strategy of Jonsdottir et al,⁴⁵ who propose desensitizing horses through intralymphatic injection of small doses of allergens in adjuvants.

Despite a missing correlation between allergen-specific IgE levels and IBH severity, we showed that the severity of IBH disease correlated well with blood eosinophil counts. Especially in the setting of progressed IBH, eosinophils seemed to dominate the allergic reaction. The eosinophilia is caused by a late phase of type I and delayed-type allergic responses (type IV) to insect bites. This is in contrast to insect venom allergy in human subjects, in which eosinophilia has not been described. Thus eosinophil-dependent IBH shares common aspects with human asthma, which also has an IgE component but is eosinophil driven.⁴⁶ This implies that human allergy in the lungs seems to share common aspects with equine allergy in the skin. However, atopic dermatitis of human skin is a multifactorial and complex disease with various trigger factors, only one of them being allergens. Although serum concentration of human eosinophilic cationic protein is a diagnostic marker for atopic dermatitis in

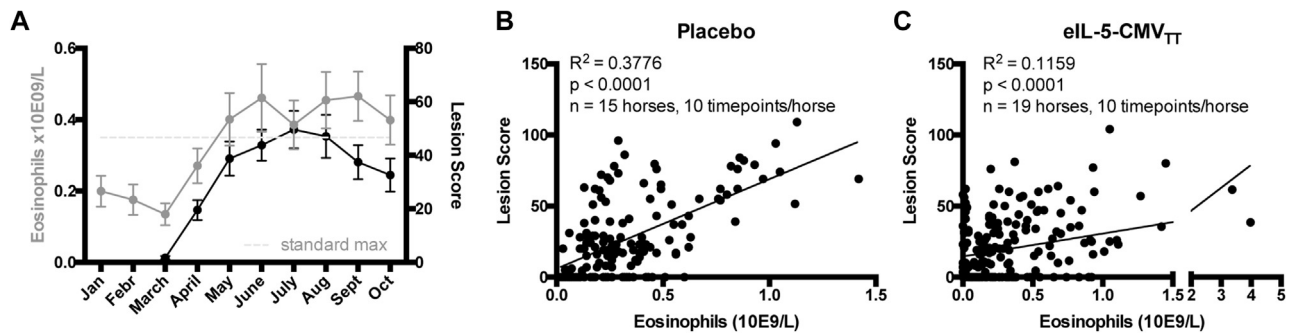


FIG 6. Interrelation between blood eosinophil counts and lesion scores. **A**, Mean blood eosinophil counts (gray symbols and line, left y-axis) and mean lesion scores (black symbol and line, right y-axis) of placebo-treated horses (n = 15) determined in the treatment season from January until October or March until October, respectively. The normal maximum blood eosinophil count is indicated by a dotted gray line. Months with more than 1 measurement show average lesion score values. **B**, Eosinophil counts versus lesion scores of all 15 placebo-treated horses for 10 time points from each horse. **C**, Eosinophil counts versus lesion scores of all 19 vaccinated horses for 10 time points from each horse.

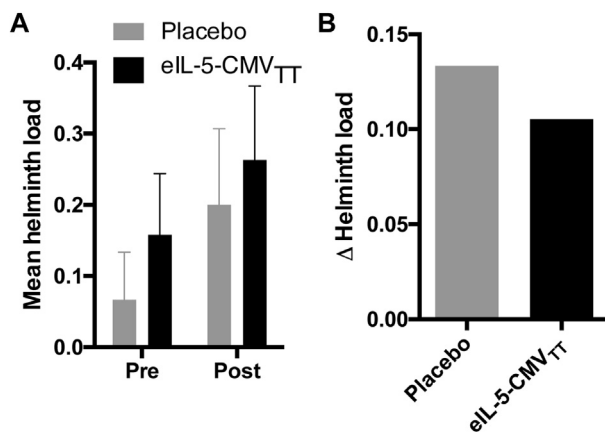


FIG 7. Side effect: parasitic load. **A**, Mean helminth burden in placebo-treated horses (gray columns, n = 15) and eIL-5-CMV_{TT}-vaccinated horses (black columns, n = 19). No measureable helminths in excrement = 0, detected helminths in excrement = 1. Excrement was taken in the treatment season before injections (pre) and at the study's end in autumn (post). **B**, Average seasonal change in helminth burden for placebo-treated (gray column) and vaccinated horses (black column) was calculated by subtracting the mean value determined at the beginning from that at the end of the treatment season.

human subjects,⁴⁷ eosinophilia is not suggested to trigger dermatitis in human subjects.⁴⁸ Therefore we think that human asthma is the correlate to equine IBH, although the affected organs differ.

IL-5 is a very well-validated target, and abundant data in mice and human subjects indicate that blocking IL-5 is a safe and efficacious way to reduce eosinophil-mediated inflammation. Therefore IL-5 might be an ideal target for safe IBH therapy in horses. Two different anti-human IL-5 antibodies have been developed for the treatment of eosinophil-associated diseases, such as hypereosinophilic syndrome or eosinophilic asthma: mepolizumab (GlaxoSmithKline, Research Triangle Park, NC) and reslizumab (Teva Pharmaceuticals, Petah Tikva, Israel). The mechanism of action for an anti-IL-5 antibody is blockage of eosinophil development, survival, and activation. Evidence from

eosinophil-deficient mouse models and mAb studies in human subjects suggests that eosinophil depletion has a negligible effect on immunity to infection and other essential host functions.^{26,28,49} Moreover, IL-5 is classified as a lineage-specific cytokine for eosinophils only and has a central role in the differentiation, activation, and survival of eosinophils exclusively. Hence depletion of this cytokine is expected to have limited negative effects on other lineages than eosinophils. Therefore a treatment that specifically targets eosinophils seems to have limited effects on the host. Indeed, no safety issues were reported in any of the human trials or after-market authorization.^{49,50} In line with these findings, eIL-5-CMV_{TT} vaccine was well tolerated in horses and did not predispose to increased infections. Specifically, and given the fact that eosinophils are known to have antiparasitic effects and horses are natural hosts of certain intestinal helminths, no difference in helminth burden was found during the study period when comparing vaccinated and placebo-treated horses. However, more detailed safety studies will be required because of limitations of our placebo-controlled randomized clinical study, including the number of horses and the facts that we only included Icelandic horses and do not know yet the effect on helminth burden during long-term treatment.

Two injections of eIL-5-CMV_{TT} formulated in PBS induced high levels of anti-self antibodies recognizing eIL-5. Vaccine booster immunizations maintained these levels, whereas antibody responses decreased in the absence of further injections. Hence vaccination against IL-5 induced high levels of antibodies that were reversible in the absence of booster injections. High antibody titers were also measured against CMV_{TT}-VLPs and were used as a surrogate marker of vaccination because the immune system readily induces antibodies against the foreign CMV particle. Responses against IL-5 were slightly more variable than responses against the carrier VLPs, but responses against IL-5 and the CMV carrier correlated well overall. This is consistent with observations in human subjects immunized against IL-1 β and might indicate that the tightly packed VLP surface is able to activate the immune system more consistently than the IL-5 covalently attached to VLPs.⁵¹ Furthermore, and in contrast to anti-CMV antibodies, anti-IL-5 antibodies can bind to host IL-5 in the serum, and thus antibodies involved in

antigen-antibody complexes might no longer be detectable in the ELISA performed in this study. Natural variations in IL-5 levels between subjects will perhaps lead to different rates of depletion of anti-IL-5 antibodies and hence provide a perception of variability in anti-IL-5 antibody responses compared with CMV.

Similarly, as seen with anti-IL-5 mAbs in human subjects, we showed that vaccine-induced anti-IL-5 autoantibodies in horses were able to treat eosinophil-mediated symptoms. Clinical scores improved in eIL-5-CMV_{TT}-vaccinated horses when compared with the previous season of the same horses and placebo-treated horses of the same season. One major advantage of vaccines over mAbs is that there is no induction of antitherapeutic antibodies by the host because these polyclonal vaccine-induced antibodies are self-made. This ensures that the therapeutic vaccination can be given each year before the IBH season starts, leading to high antibody titers during the disease period and decreasing antibody titers during the unaffected time of year. For human subjects, antibodies induced against mAbs are a tangible problem that can limit the choice of therapeutics, an issue commonly seen in certain mAbs (eg, infliximab).^{52,53} Along these lines, to date, it is unclear how inbred the different horses are with respect to antibody allotypes. Therefore immunogenicity of the different horse breeds will be difficult to predict.

Another advantage of the vaccine's approach is the low cost of goods because much lower amounts of protein are needed for immunization compared with mAbs, which are usually applied based on body weight. Replacing the costly monoclonal therapies with active vaccination might also be an attractive treatment modality in human subjects, providing relief to health insurance systems. Therefore it would be interesting and also worthwhile to compare an anti-human IL-5 mAb versus human IL-5-VLP vaccines in human subjects.

Blood eosinophil levels during an IBH season increased typically in the spring and reached a peak in June. IBH lesion development followed eosinophil counts, with some delay. Overall, eosinophil counts in the blood of placebo-treated and unmanipulated horses correlated well with skin lesion scores, suggesting that in an unbiased system eosinophil levels in blood are a good indicator of eosinophil counts in skin and lesion severity. However, this correlation was lost in horses vaccinated against eIL-5, most likely because eosinophils do not efficiently enter tissues in the absence of IL-5. Of note, in the follow-up year of this study, during which slightly greater anti-IL-5 responses were induced, a statistically significant reduction of eosinophils in blood was observed (unpublished data). An alternative explanation for the absence of a correlation between blood eosinophil counts and antibody titers in the present study might be that the vaccine in the current vaccine preparation contained free non-coupled IL-5, whereas in the follow-up study this free IL-5 has been removed.

Taken together, eIL-5-CMV_{TT} successfully induced autoantibodies and mediated a statistically significant reduction in lesion scores in vaccinated horses when compared with placebo-treated horses and the pretreatment season. This is the first time that an anticytokine vaccine has shown clinical efficacy for the treatment of a disease in the target species and might facilitate the development of a similar vaccine targeting IL-5 in human subjects.

We thank all the horse owners who participated with their horses in our clinical studies. We thank Michael Fontaine and Hans-Uwe Simon for helpful discussion.

Clinical implications: Long-term treatment of horses affected by recurrent insect-bite hypersensitivity might be possible by using a vaccine targeting IL-5 and thus limiting eosinophil development and recruitment to the affected organ skin.

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METHODS

MALDI-MS/MS and HPLC of digested eIL-5–C-His

MALDI-MS/MS and HPLC of digested eIL-5–C-His were performed by the Functional Genomic Center, Zurich. eIL-5 was freshly digested with 1 µg of trypsin (20:1) in 0.1% TFA/PBS buffer, pH 5 to 6, and analyzed by using 2

different HPLC columns (C8 and C18). Samples were mixed 1:1 with matrix solution (1.4 mg/mL alpha-4-hydroxycinnamic acid in 85% cerium nitrate, 0.1% trifluoroacetic acid, and 1 mmol/L MALDI-MS/MS and HPLC of digested eIL-5–C-His $\text{NH}_4\text{H}_2\text{PO}_4$) and spotted on the target. MALDI-MS/MS measurements were performed manually.

[SCORE SHEET LESION GRADING]										1/4
STUDY TITLE		„Therapeutic Vaccination against Equine Insect Bite Hypersensitivity (IBH) – Impfung gegen Sommerkeizem“								
STUDY ID										
DATE, PLACE										
OWNER										
HORSE NAME / PATIENT ID										
<p>Points are given for each summer eczema site. Every site is judged for intensity on location, size/diameter, blood, hairloss, scales, crust and lichenification. Per site intensities are graded from 1 to maximal 4 points. Total points is sum of all points. For more information see page 4.</p>										
LOCATION										
		UP	MIDDLE	DOWN	LIGHT	STRONG	POINTS			
TAIL	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
MANE	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
BELLY	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
WITHERS	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
FLANK	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
FACE	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
EAR	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
LEGS	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
CROUP	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
OTHER	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	_____			
SIZE, DIAMETER										
							POINTS			
TAIL	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
MANE	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
BELLY	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
WITHERS	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
FLANK	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
FACE	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
EAR	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
LEGS	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
CROUP	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
OTHER	< 0.5 cm <input type="checkbox"/>	0.5 ≤ x < 1 cm <input type="checkbox"/>	1 ≤ x < 2 cm <input type="checkbox"/>	≥ 2 cm <input type="checkbox"/>			_____			
<p>Antonia Fetteleschoss 2014, updated 2015</p>										

[SCORE SHEET LESION GRADING]										2/4
BLOOD										
							POINTS			
TAIL	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
MANE	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
BELLY	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
WITHERS	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
FLANK	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
FACE	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
EAR	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
LEGS	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
CROUP	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
OTHER	INTACT EPIDERMIS <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
HAIRLOSS										
							POINTS			
TAIL	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
MANE	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
BELLY	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
WITHERS	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
FLANK	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
FACE	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
EAR	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
LEGS	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
CROUP	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
OTHER	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	NO HAIR <input type="checkbox"/>	_____					
SCALES										
							POINTS			
TAIL	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					
MANE	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					
BELLY	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					
WITHERS	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					
FLANK	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					
FACE	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					
EAR	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					
LEGS	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					
CROUP	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					
OTHER	NONE <input type="checkbox"/>	TINY, FEW <input type="checkbox"/>	MODERATE, MID-SIZE <input type="checkbox"/>	MANY, BIG <input type="checkbox"/>	_____					

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[SCORE SHEET LESION GRADING]										3/4
CRUST										
							POINTS			
TAIL	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
MANE	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
BELLY	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
WITHERS	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
FLANK	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
FACE	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
EAR	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
LEGS	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
CROUP	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
OTHER	NONE <input type="checkbox"/>	TINY <input type="checkbox"/>	HALF <input type="checkbox"/>	TOTAL <input type="checkbox"/>	_____					
LICHENIFICATION / SWELLING										
							POINTS			
TAIL	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
MANE	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
BELLY	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
WITHERS	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
FLANK	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
FACE	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
EAR	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
LEGS	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
CROUP	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
OTHER	NONE <input type="checkbox"/>	MILD <input type="checkbox"/>	MODERATE <input type="checkbox"/>	SEVERE <input type="checkbox"/>	_____					
SHEATH / UDDER										
							POINTS			
SHEATH	GRADE 1 <input type="checkbox"/>	GRADE 2 <input type="checkbox"/>	GRADE 3 <input type="checkbox"/>	GRADE 4 <input type="checkbox"/>	_____					
UDDER	GRADE 1 <input type="checkbox"/>	GRADE 2 <input type="checkbox"/>	GRADE 3 <input type="checkbox"/>	GRADE 4 <input type="checkbox"/>	_____					
TOTAL POINTS										

NOTES										

VETERINARY _____ RESEARCHER _____										
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[SCORE SHEET LESION GRADING]										4/4
LESION SCORING										
<p>For IBH symptom scoring, the location (tail, mane, belly, flank, face, ear, leg, and the like) where IBH lesions occur are recorded. Each location is divided into 3 parts: up, middle, down. Further according to the number of lesions, each location is classified into light and strong. Dependent on how many parts are affected (up / middle / down) and how many lesions per location are found (light / strong), 1 to 4 points can be scored (1 point = one part affected, lesion light; 4 points = all three parts affected, lesion strong).</p> <p>Moreover, these locations are classified for 6 further properties: size (diameter), blood, hair loss, scales, crust, and lichenification / swelling. For all these properties also 1 to 4 points can be scored. Size is divided into < 0.5 cm (1 point), 0.5 ≤ x < 1 cm (2 points), 1 ≤ x < 2 cm (3 points), and ≥ 2 cm (4 points). Blood is divided into intact epidermis (1 point), mild (2 points), moderate (3 points), and severe (4 points). Hair loss is divided into mild (1 point), moderate (2 points), severe (3 points), and no hair (4 points). Scales is divided into none (1 point), tiny, few (2 points), moderate, mid-size (3 points), and many, big (4 points). Crust is divided into none (1 point), tiny (2 points), half (3 points), and total (4 points). Lichenification and/or swelling is divided into none (1 point), mild (2 points), moderate (3 points), and severe (4 points).</p> <p>Additionally, if sheath or udder is swollen, minimally 5 or maximally 20 points can be scored: grade 1 (5 points), grade 2 (10 points), grade 3 (15 points), and grade 4 (20 points).</p> <p>Finally all points are added up and are the IBH symptom score.</p>										

FIG E1. Score sheet lesion scoring. Results are as described in the [Methods](#) section of the main article.

